Chemistry & Biology
Article

# Directed Evolution of a Lysosomal Enzyme with Enhanced Activity at Neutral pH by Mammalian Cell-Surface Display

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DOI 10.1016/j.chembiol.2008.10.008

## SUMMARY

**Human β-glucuronidase, due to low intrinsic immunogenicity in humans, is an attractive enzyme for tumor-specific prodrug activation, but its utility is hindered by low activity at physiological pH. Here we describe the development of a high-throughput screening procedure for enzymatic activity based on the stable retention of fluorescent reaction product in mammalian cells expressing properly folded glycoproteins on their surface. We utilized this procedure on error-prone PCR and saturation mutagenesis libraries to isolate β-glucuronidase tetramers that were up to 60-fold more active ($k_{cat}/K_m$) at pH 7.0 and were up to an order of magnitude more effective at catalyzing the conversion of two structurally disparate glucuronide prodrugs to anticancer agents. The screening procedure described here can facilitate investigation of eukaryotic enzymes requiring posttranslational modifications for biological activity.**

## INTRODUCTION

Chemotherapy is an important treatment modality for advanced cancers, but systemic toxicity typically limits therapeutic efficacy. Antibody- (Bagshawe et al., 1988; Senter et al., 1988) and gene-directed enzyme prodrug therapy (Chen et al., 1994) (ADEPT and GDEPT) are approaches to increase the therapeutic index of cancer chemotherapy. ADEPT and GDEPT employ antibody-targeted or gene-expressed enzymes to preferentially activate nontoxic antineoplastic drugs at cancer cells, thereby sparing normal tissues from high concentrations of toxic drug. Although microbial and viral enzymes are largely utilized for targeted prodrug activation, the development of strong immune responses against foreign proteins can rapidly hinder therapeutic effectiveness (Napier et al., 2000; Sharma et al., 1992). The utilization of human enzymes is therefore highly desirable to minimize immune responses and allow multiple rounds of treatment.

β-glucuronidase (βG, EC 3.2.1.31) is an attractive enzyme for ADEPT and GDEPT because both mammalian and bacterial homologs exist. A variety of glucuronide prodrugs have demonstrated impressive antitumor activity in diverse tumor models (de Graaf et al., 2002). Although many studies have employed *Escherichia coli* β-glucuronidase (eβG) to activate glucuronide prodrugs at cancer cells because of the high specific activity of the bacterial enzyme (Chen et al., 2001, 2007; Cheng et al., 1999b; Wang, 1992), development of human β-glucuronidase (hβG) therapies is desirable to minimize immune responses in patients. hβG is sequestered in lysosomes and is therefore largely inaccessible to membrane-impermeable glucuronides, minimizing the problem of systemic activation of prodrugs by endogenous enzymes (Cheng et al., 1999a). As an acid hydrolase, hβG displays maximum activity at pH 4.0, but relatively low activity at neutral pH (Chen et al., 2007). Translation of glucuronide prodrug therapy to the clinic could be facilitated by generating hβG variants that more efficiently hydrolyze prodrugs under the physiological conditions present in tumors.

The pH profile of enzymes has been altered by substituting specific amino acids that directly (Kim et al., 2006) or indirectly (Makde et al., 2006) contact substrates as well as by changing amino acids on the enzyme surface (Russell and Fersht, 1987). However, it is difficult to rationally alter the pH profile of an enzyme with retention of catalytic activity. Directed evolution, on the other hand, is an extremely versatile and powerful method to modify protein properties without a priori detailed knowledge of protein structure-function relationships (Kaur and Sharma, 2006).

Whereas most directed evolution has been performed in bacteria, many eukaryotic proteins require posttranslational modifications such as glycosylation for proper folding and catalytic activity. For example, hβG is expressed as a tetramer composed of four identical monomeric subunits of 629 amino acids, each possessing four N-linked oligosaccharides that are required for proper protein folding (Shipley et al., 1993). Furthermore, assay of hβG activity at defined pH values is difficult due to cell-to-cell variations in lysosomal pH (Anderson and Orci, 1988). In the present study, we solved these problems by tethering hβG on the surface of mammalian cells to allow assay of properly glycosylated and active enzyme under defined conditions. We

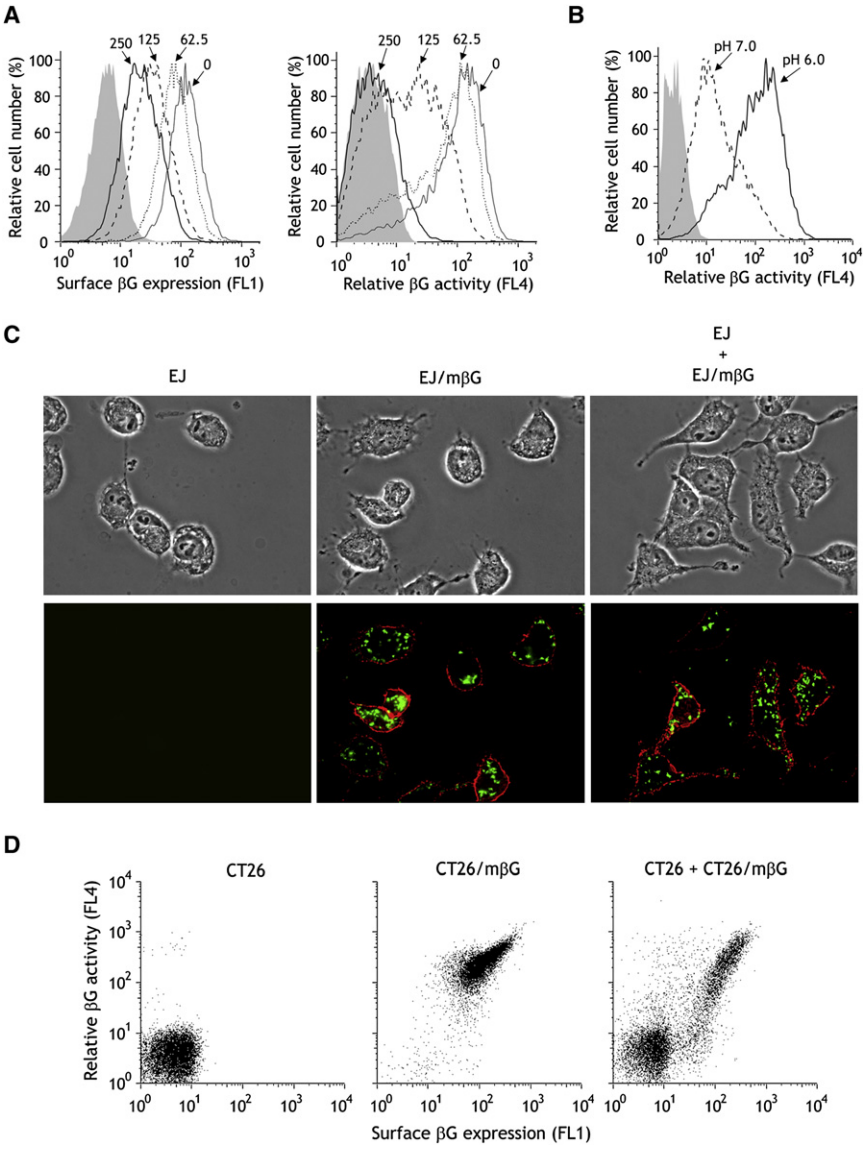


**Figure 1. Characterization of Membrane-Tethered βG**

(A) 3T3 cells that stably expressed membrane-tethered hβG were treated with 250 (bold solid line), 125 (dashed line), 62.5 (dotted line), or 0 (regular solid line) μg/ml trypsin for 5 min at 37°C before 100 μM ELF-97 β-D-glucuronide was added to the cells at pH 6.0 for 15 min at 37°C. Surface hβG expression was detected by staining with 7G8-FITC. The cells were analyzed for FITC (surface hβG expression) or ELF-97 alcohol (hβG enzymatic activity) fluorescence on a flow cytometer. Negative control (3T3) cells are shown in gray.

(B) CT26/mβG cells were stained with 25 μM ELF-97 β-D-glucuronide at pH 6.0 (solid line) or pH 7.0 (dashed line) for 5 min at room temperature. Negative control CT26 cells were stained with ELF-97 β-D-glucuronide at pH 6 (gray).

(C) EJ and EJ/mβG cells were seeded separately (left and middle panels) or mixed (right panel) on cover slides before staining for βG activity (with 100 μM ELF-97 β-D-glucuronide, green) and membrane-anchored mβG expression (with biotin-labeled goat anti-HA followed by streptavidin-labeled rhodamine, red). Cells were observed under a fluorescence microscope equipped with a CCD detector.

(D) CT26 and CT26/mβG cells were stained individually or in a 50/50 mixture for βG activity (25 μM ELF-97 β-D-glucuronide [pH 6.0] at room temperature for 5 min) and mβG protein expression (mAb 7G7 and goat anti-rat-FITC) and then analyzed by FACS.

show that combination of mammalian cell-surface display with high-throughput flow cytometric sorting allows successful isolation of hβG variants with enhanced activity at neutral pH.

## RESULTS

### Development of a Mammalian Cell-Surface-Tethered Enzyme Screening System

We tethered human βG (hβG) and mouse βG (mβG) on the surface of mammalian cells by fusing the cDNA for the respective βG genes to the juxtamembrane Ig-like extracellular domain, transmembrane domain, and cytoplasmic tail of murine B7-1 (Chen et al., 2007; Cheng and Roffler, 2008). mβG was employed for some assays because it displays about 3-fold greater enzymatic activity than hβG at pH 7.0, allowing better sensitivity for initial method development. hβG was stably expressed with retention of enzymatic activity on 3T3 fibroblasts, as determined by surface immunofluorescence staining with a FITC-labeled anti-hβG antibody (7G8-FITC) and by hydrolysis of the substrate ELF-97 β-D-glucuronide to ELF-97 alcohol (Telford et al., 2001), a fluorescent product that remained associated with the cells (Figure 1A). Trypsin proteolysis of cell-surface proteins reduced hβG expression and enzymatic activity in parallel (Figure 1A), demonstrating that membrane-tethered hβG was responsible for hydrolysis of ELF-97 β-D-glucuronide. The enzymatic activity of membrane-tethered mβG depended on the extracellular pH, allowing convenient control of the enzyme environment (Figure 1B). Cells expressing membrane-tethered mβG were clearly distinguishable from wild-type (WT) cells under fluorescence illumination after staining mixed-cell populations with ELF-97 β-D-glucuronide (Figure 1C). To determine whether high-throughput flow cytometric sorting of cells based on relative βG activity was feasible, a mixture of WT cells and cells expressing membrane-tethered mβG was first stained with rat anti-mβG antibody followed by goat anti-rat FITC conjugate and then incubated with ELF-97 β-D-glucuronide. Two discrete populations of cells could be clearly separated (Figure 1D), demonstrating that enzymatically generated ELF-97 alcohol remained associated with cells expressing membrane-tethered βG during the staining and analysis procedure.

### Selection of hβG Variants with Enhanced Activity at Neutral pH

Based on these results, we developed a robust high-throughput fluorescence-activated cell-sorting method to isolate hβG mutants with enhanced activity at neutral pH (Figure 2A). We employed error-prone PCR to generate a cDNA library (EP1 library) with a diversity of ~5 × $10^6$ containing an average of 4.5 amino acid mutations per hβG gene. 3T3 fibroblasts were infected at low MOI (multiplicity of infection; ~0.6 cfu/cell) with a VSV-G pseudotyped retroviral virus library to generate ~$10^7$ independent 3T3 clones. After selection in G418, viable cells were stained with 7G8-FITC (anti-hβG-FITC conjugate) and incubated with ELF-97 β-D-glucuronide before they were sorted on a flow cytometer for enhanced βG activity relative to hβG expression levels. The first round of screening was performed at pH 5.0 to enrich for rare cells displaying enhanced βG enzymatic activity at slightly elevated pH (Figure 2B, left panel). Selected cells (~0.5% of the total cell population) were cultured to expand their numbers and then resorted two additional times after immunofluorescence staining for hβG expression with 7G8-FITC and labeling βG activity with ELF-97 β-D-glucuronide at pH 6.5 to isolate cells displaying enhanced surface enzymatic activity at elevated pH. The sorted population displayed increased enzymatic activity as compared to cells expressing membrane-tethered WT hβG (Figure 2B, right panel). The sorted population also exhibited enhanced enzyme activity at both pH 5.0 and pH 6.5, indicating that enzyme variants with a broader pH profile can be isolated by applying selection pressure based on reaction pH (Figure 2C). Sequencing the hβG gene in 12 individual cell clones revealed multiple mutations. However, all hβG variants had mutations at position 545 (50% T→A and 50% T→S). To determine the effect of amino acid substitutions at position 545 on enzyme activity, the single amino acid mutants T545A (E1-A), T545G (E1-G), T545S (E1-S), and T545Y (E1-Y) were generated and the corresponding enzymes were purified from the culture medium of stably transfected fibroblasts. Assay for enzymatic activity at pH 7.0 with the substrate *p*-nitrophenol β-D-glucuronide revealed that E1-G and E1-A displayed about 2.5-fold greater activity than WT hβG, E1-S exhibited about 1.5-fold greater activity, and E1-Y was almost inactive (data not shown). Thus, single amino acid changes can affect hβG activity at neutral pH. We employed the E1-G (50%) and E1-A (50%) variants as starting material to generate an error-prone cDNA library (EP2) with 5 × $10^6$ members containing an average of 2.6 amino acid mutations per hβG gene. Independent 3T3 clones (5 × $10^7$) were immunofluorescence stained with 7G8-FITC and reacted with ELF-97 β-D-glucuronide at pH 7.0. Sixteen individual cell clones were isolated after three rounds of sorting and the hβG genes were sequenced. All of the isolated hβG genes possessed identical amino acid substitutions at position 255 (L→Q) and 545 (T→G) with variable substitutions at other positions. One high-activity variant, termed E2-20, was selected for further examination (Table 1).

To help differentiate between beneficial and null mutations in the hβG variants, hβG cDNA isolated from the sorted EP1 library (30%) was shuffled with the WT hβG gene (70%) to breed out null mutations. The shuffled backcross library was then expressed on 3T3 fibroblasts and screened with 7G8-FITC and ELF-97 β-D-glucuronide at pH 7.0. After one round of flow cytometric sorting, individual cell clones were isolated and the hβG gene was sequenced. Among 13 single-cell clones, amino acid changes at positions 545, 595, and 599 were consistently associated with increased hβG activity at neutral pH values.

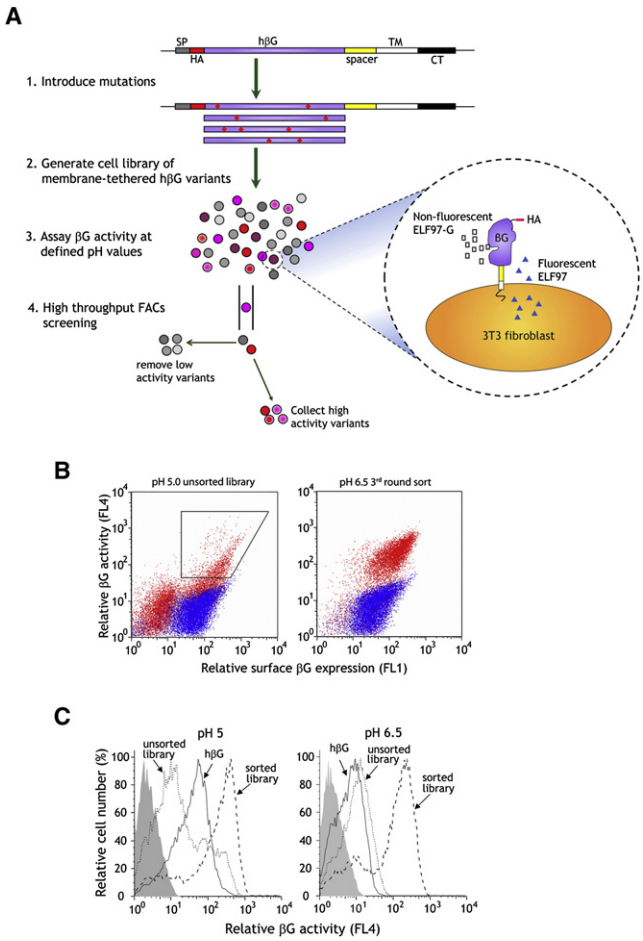


**Figure 2. High-Throughput FACS Screening at Defined pH**

(A) The transgene codes for the immunoglobulin κ chain signal peptide (SP), an HA epitope (HA), hβG fused to the first extracellular domain (spacer), transmembrane domain (TM), and cytoplasmic tail (CT) of the murine B7-1 antigen. (1) Mutations were introduced in the hβG gene by error-prone PCR, DNA shuffling, or saturation mutagenesis. (2) hβG transgenes were retrovirally infected in 3T3 cells to generate a membrane-tethered hβG cell library. (3) Surface expression of hβG allowed adjustment of reaction pH for screening. The cell library was reacted with ELF-97 β-D-glucuronide to accumulate fluorescent ELF-97 alcohol in cells. (4) Cells exhibiting higher βG activity were collected and expanded for further analysis.

(B) 3T3 cells expressing membrane-tethered WT (blue) or mutant (red) hβG were reacted with 25 μM ELF-97 β-D-glucuronide for 15 min at room temperature and then stained with 7G8-FITC. Cells that exhibited higher βG activity at pH 5 were isolated by FACS (left panel). After two additional rounds of sorting at pH 6.5, a population of cells with higher βG activity at pH 6.5 was isolated (right panel).

(C) 3T3 fibroblasts expressing WT hβG (solid line), or unsorted (dotted line) or sorted (dashed line) collections of hβG variants, were exposed to 25 μM ELF-97 β-D-glucuronide at pH 5 (left panel) or pH 6.5 (right panel) for 15 min at room temperature. Negative control (3T3) cells are shown in gray.

**Table 1. Amino Acid Substitutions in Selected hβG Variants**

| Clone | Amino Acid Residue 159 | 243 | 255 | 518 | 545 | 595 | 599 | Relative Activity[a] |
|---|---|---|---|---|---|---|---|---|
| Wild-type | V | L | L | Q | T | E | T | 1 |
| E1-S | | | | | S | | | 21 |
| E1-G | | | | | G | | | ND |
| S2 | M | | | | S | L | L | 65 |
| S28 | | | | R | S | Y | F | 47 |
| E2-20 | | Q | Q | | G | | | 116 |

ND, not determined.

[a] 3T3 fibroblasts expressing membrane-tethered forms of the indicated hβG variants were assayed for hβG surface expression and βG enzymatic activity. Relative activity of membrane-tethered hβGs was calculated as the ratio of FL4 (hβG activity)/FL1 (surface hβG expression) at pH 7.0 and normalized to the FL4/FL1 of WT hβG. The substrate was ELF-97 β-D-glucuronide.

## Saturation Mutagenesis

To further examine the influence of varying the amino acids at positions 545, 595, and 599 on the catalytic activity of hβG, we employed saturation mutagenesis to generate a small library ($\sim 10^5$ cell clones) covering all possible combinations of amino acids at these three sites. The library was screened by two rounds of cell sorting at pH 7.0. Among 14 single-cell clones displaying high activity, seven different amino acids (all nonnegatively charged) appeared at position 595. Furthermore, 79% of the hβG variants had serine at position 545, and 13 of 14 clones contained amino acids with hydrophobic side groups (28.5% leucine, 28.5% isoleucine, and 43% phenylalanine) at position 599. Two variants (S2 and S28 in Table 1) were selected for further analysis.

## Characterization of βG Variants

Table 1 summarizes the mutations present in five membrane-tethered hβG variants which displayed 20- to 115-fold greater enzyme activity than WT hβG for ELF-97 β-D-glucuronide at pH 7.0. The amount of hβG protein expressed by each cell line varied as determined by immunoblot analysis (see Figure S1A available online). There was a strong linear correlation between total hβG expression level (as determined by hβG band intensity on the immunoblot in Figure S1A) and surface hβG levels, as determined by immunofluorescence staining of hβG followed by flow cytometric analysis (Figure S1B). This confirms that immunofluorescence staining allows convenient normalization of enzyme activities for differences in hβG expression levels.

To further characterize the hβG variants, we purified soluble hβG enzymes from 3T3 producer cells (Figure 3A). The proteins migrated slower than the predicted molecular weight of an hβG monomer due to the presence of N-linked oligosaccharides in the enzyme (Shipley et al., 1993). hβG displayed maximal activity at pH 4.0 but only 2% of maximal activity at pH 7.0 (Figure 3B). The hβG variants exhibited maximal activity at pH 4.5 with relatively broad pH profiles. The kinetic properties of hβG and variants were compared at pH 4.5 and pH 7.0 (Table 2). Consistent with enhanced enzymatic activity of membrane-tethered hβG, the soluble hβG variants displayed enhanced $k_{cat}/K_m$ values at both pH 4.5 and 7.0 (Figure 3C). At pH 7.0, E1-S and E1-G displayed $k_{cat}/K_m$ values 4- and 10-fold higher than WT hβG, whereas S2, S28, and E2-20 variants displayed 20- to 60-fold enhancements in $k_{cat}/K_m$. Surprisingly, the $k_{cat}/K_m$ values of the S2 and E2-20 enzymes at pH 7.0 were about 2-fold greater than the $k_{cat}/K_m$ of WT hβG at pH 4.5. The dramatic increases in substrate affinity at acidic and neutral pH explain the overall enhancement in the activities of the hβG variants. Changes in amino acid sequence can adversely affect protein stability (Smith et al., 1997). However, the enzymatic activity of recombinant hβG variants at pH 7.0 was fully retained for at least 2 weeks

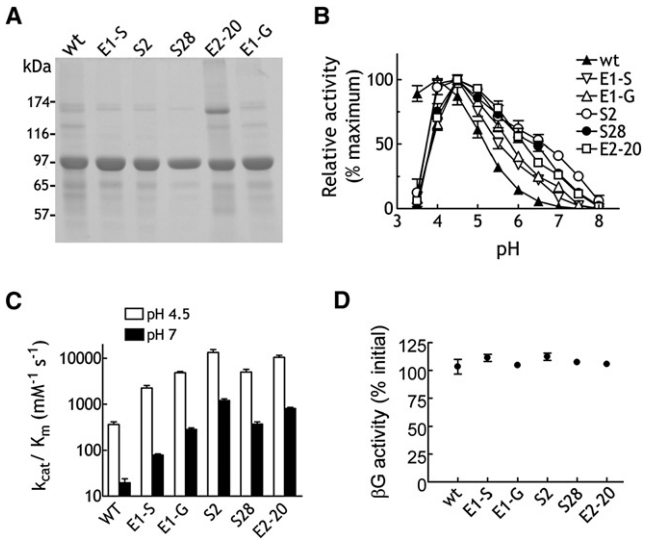


**Figure 3. pH-Dependent Activities of Soluble hβG Variants**

(A) SDS-PAGE of purified WT and variant hβG proteins. Proteins were visualized by staining with Coomassie blue G-250.

(B) The relative enzymatic activities (percentage of maximum activity using 0.5 mM 4-methylumbelliferyl β-D-glucuronide at 37°C for 30 min, normalized to the maximum activity of each clone) of the recombinant enzymes at the indicated pH values are shown (n = 3). Error bars, SD.

(C) Purified hβG proteins were reacted with various concentrations of ELF-97 β-D-glucuronide at pH 4.5 and 7.0 to determine their kinetic constants. The $k_{cat}/K_m$ values at pH 4.5 and 7.0 are shown (n = 3). Error bars, SD.

(D) Percentage of original enzymatic activity retained by hβG proteins after incubation for 14 days at 37°C, pH 7.0. The activity was measured by the hydrolysis of 0.5 mM 4-methylumbelliferyl β-D-glucuronide at 37°C for 30 min. Error bars, SEM.

**Table 2. Kinetic Parameters of Wild-Type and hβG Variants at pH 4.5 and 7.0**

| Enzyme | pH 4.5 $K_m$ (mM) | pH 4.5 $k_{cat}$ ($s^{-1}$) | pH 7.0 $K_m$ (mM) | pH 7.0 $k_{cat}$ ($s^{-1}$) |
|---|---|---|---|---|
| Wild-type | 0.48 ± 0.06 | 175 ± 17 | 2.25 ± 0.67 | 41 ± 6 |
| E1-S | 0.052 ± 0.004 | 117 ± 14 | 0.39 ± 0.04 | 31 ± 6 |
| E1-G | 0.022 ± 0.002 | 106 ± 4 | 0.11 ± 0.01 | 32 ± 4 |
| S2 | 0.0081 ± 0.0008 | 107 ± 7 | 0.035 ± 0.003 | 42 ± 4 |
| S28 | 0.016 ± 0.003 | 79 ± 3 | 0.072 ± 0.024 | 26 ± 3 |
| E2-20 | 0.0067 ± 0.0005 | 71 ± 3 | 0.038 ± 0.005 | 30 ± 5 |

Results represent mean values of triplicate determinations ± SD. The substrate was ELF-97 β-D-glucuronide.

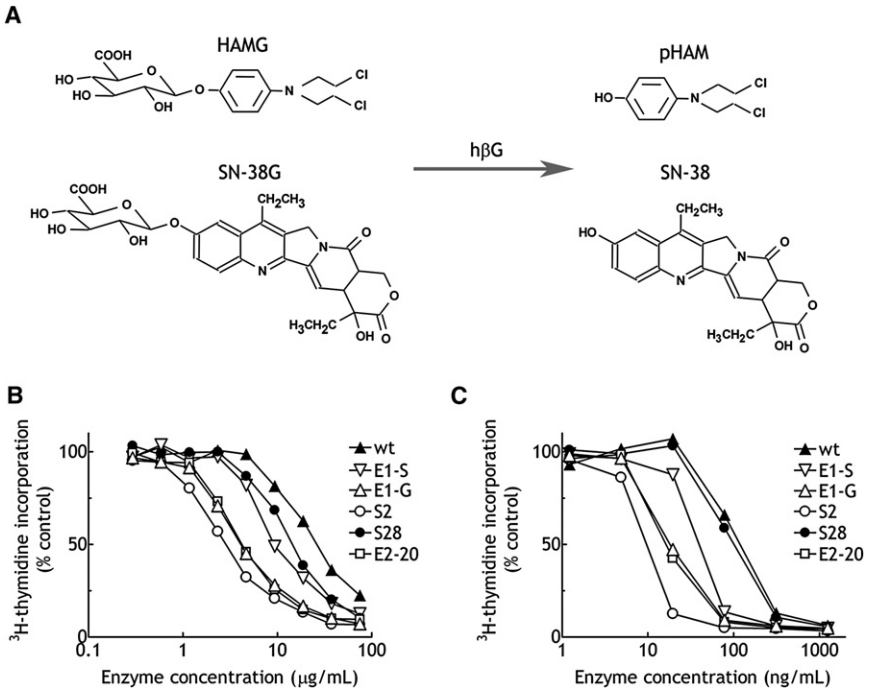


**Figure 4. Comparison of Prodrug Activation by Wild-Type and Mutant hβGs**

(A) Prodrug HAMG or SN-38G is hydrolyzed specifically by hβG to cytotoxic pHAM or SN-38.

(B and C) EJ human bladder cancer cells were incubated with graded concentrations of hβG variants in RPMI (pH 6.8) containing either (B) 10 μM HAMG or (C) 100 nM SN-38G for 24 hr. The cells were further incubated for 24 hr in fresh medium before incorporation of [$^3$H]thymidine into cellular DNA was measured.

at 37°C, indicating that the mutant enzymes displayed good stability at neutral pH (Figure 3D).

### Prodrug Activation by hβG Variants

To determine whether the hβG variants also exhibited enhanced activity for anticancer glucuronide prodrugs, we incubated EJ human bladder cancer cells with a constant concentration of HAMG, a nontoxic glucuronide prodrug of *p*-hydroxyaniline mustard, and graded concentrations of WT or mutant hβGs. All hβG variants hydrolyzed HAMG to cytotoxic drug more efficiently than WT hβG, as demonstrated by enhanced killing of EJ cancer cells (Figure 4A). The most active S2 variant was 9-fold more effective than WT hβG ($EC_{50}$ = 2.7 versus 24 μg/ml, respectively). A similar result was found for SN-38G, which releases the potent topoisomerase I poison SN38 (Rivory, 1996) upon hydrolysis of the glucuronide moiety (Figure 4B). The $EC_{50}$ of WT hβG and the S2 variant were 96 and 8.4 ng/ml, respectively, representing an 11-fold improvement in prodrug activation.

## DISCUSSION

Alteration of the pH profile of mammalian enzymes by directed evolution has not been described to our knowledge, likely due to the difficulty in establishing an appropriate high-throughput screening system. By combining error-prone PCR with a high-throughput mammalian cell-surface-tethered screening system, we successfully generated hβG variants with enhanced activity over an extended pH range. Importantly, the hβG variants more effectively hydrolyzed two structurally distinct anticancer glucuronide prodrugs at neutral pH. Because previous studies have demonstrated that antibody-hβG immunoenzymes can selectively activate glucuronide prodrugs in tumor xenografts to produce strong antitumor activity (Bosslet et al., 1994), the hβG variants developed here are anticipated to further increase the therapeutic efficacy of glucuronide prodrug therapy. hβG variants with increased catalytic activity at acidic pH may also be of interest for enzyme replacement therapy of patients suffering from Sly disease (mucopolysaccharidosis type VII), especially for organs, such as the brain, which are difficult to target (Grubb et al., 2008).

ADEPT and GDEPT are being actively developed to increase the therapeutic index of cancer chemotherapy. In ADEPT, an antibody-enzyme conjugate or fusion protein is allowed to accumulate at cancer cells before a relatively nontoxic prodrug is administered. Selective enzymatic hydrolysis of prodrug at cancer cells provides high localized concentrations of cytotoxic agent in tumors (Svensson et al., 1995). GDEPT is analogous, except that the prodrug-activating enzyme is expressed at the tumor site after viral or nonviral gene transfer of the cancer cells. Most enzymes employed for ADEPT/GDEPT have been derived from microbial or viral sources to increase specificity and reduce systemic prodrug activation, including β-lactamase (Svensson et al., 1995), carboxypeptidase G2 (Marais et al., 1997), β-glucuronidase (Wang, 1992), thymidine kinase (Moolten, 1986), nitroreductase (Anlezark et al., 1995), and cytosine deaminase (Mullen et al., 1992). However, nonhuman enzymes can induce robust immune responses, limiting the number of treatment cycles that can be administrated to patients (Napier et al., 2000). Several strategies have been investigated to reduce enzyme immunogenicity. Immunosuppressive drugs such as cyclosporine A, cyclophosphamide, and deoxyspergualin can decrease or delay the immune response against foreign proteins (Bagshawe and Sharma, 1996). Immunosuppression, however, besides producing toxicity in patients and hindering the development of antitumor immunity generated by prodrug therapy (Chen et al., 2001), has displayed rather limited suppression of strong antibody responses against bacterial enzymes (Napier et al., 2000; Sharma et al., 1996). Genetic engineering can be employed to remove immunodominant epitopes from foreign proteins (Spencer et al., 2002). For example, this technique allowed reduced binding of preexisting antibodies against an immunodominant epitope in carboxypeptidase G2 (Mayer et al., 2004). However, the large antibody repertoire and the heterogeneity of human MHC molecules make this approach technically challenging. Catalytic antibodies that can selectively hydrolyze anticancer prodrugs have

also been developed (Abraham et al., 2007). This elegant approach can utilize humanized catalytic antibodies to reduce immunogenicity. Highly active catalytic antibodies, however, are rare. Utilization of human enzyme variants with only a few amino acid changes represents an alternative approach for clinical applications.

We successfully isolated hβG variants that displayed greatly enhanced activity at pH 7. Several factors were important for successful identification of mutant enzymes. First, high levels of hβG could be tethered to the surface of 3T3 fibroblasts using membrane-anchoring domains previously developed in our lab (Chou et al., 1999; Liao et al., 2001), allowing sensitive detection of variants with desirable properties. Second, the high infectability of fibroblasts allowed generation of large libraries (>$10^7$ clones). Third, expression of hβG on mammalian cells allowed proper glycosylation required for enzymatic activity (Shipley et al., 1993). Fourth, the reaction pH could be simply controlled by altering the buffer composition because hβG was tethered to the cell surface. Finally, utilization of ELF-97 β-D-glucuronide as the substrate resulted in good retention of fluorescent reaction product in individual clones with little cross contamination of neighboring cells. ELF-97 β-D-glucuronide appears to display similar attributes as ELF-97 phosphate, which possesses high photostability and sensitivity, an unusually large Stokes shift (~170 nm), and precipitates rapidly and remains highly localized to sites of enzyme activity (Cox and Singer, 1999; Paragas et al., 2002; Telford et al., 2001). By contrast, we observed high background and lack of specificity when we screening cell libraries with fluorescein di-β-D-glucuronide (FDGlcU) or pentafluorobenzoylamino fluorescein-di-β-D-glucuronide (PFBFDGlcU) substrates, as they yield a soluble product (fluorescein) which diffuses rapidly between cells and is therefore not suitable (Lorincz et al., 1999). Several ELF-97 substrates are commercially available, including ELF-97 N-acetylglucosaminide, ELF-97 palmitate, ELF-97 acetate, and ELF-97 phosphate (Molecular Probes). Simple synthesis of analogous substrates should allow screening by mammalian cell-surface display of almost any eukaryotic enzyme that requires secretory-pathway posttranslational modifications.

Besides allowing good control of reaction conditions, mammalian cell-based surface display combined with quantitative flow cytometric sorting has additional advantages such as (1) high-throughput ($10^7$ variants can be analyzed per hour), quantitative screening; (2) the presence of an endoplasmic reticulum quality control system to minimize protein-folding errors; (3) efficient surface localization of large oligomeric proteins, such as hβG (a 380 kDa tetramer); and (4) the opportunity to reduce host expression bias by using two-dimensional labeling. For example, we normalized enzyme activity to enzyme surface levels to ensure that observed enhancements were due to altered catalytic activity rather than to cell-to-cell differences in enzyme expression levels. Mammalian surface display thus couples genotype and phenotype while allowing control of selection conditions for evolution of even large oligomeric proteins within a living system. These attributes of mammalian surface display may complement high-throughput screening methods for selection of enzymes based on bacteria (Becker et al., 2007; Olsen et al., 2000; Varadarajan et al., 2005) and yeast (Lipovsek et al., 2007) surface display.

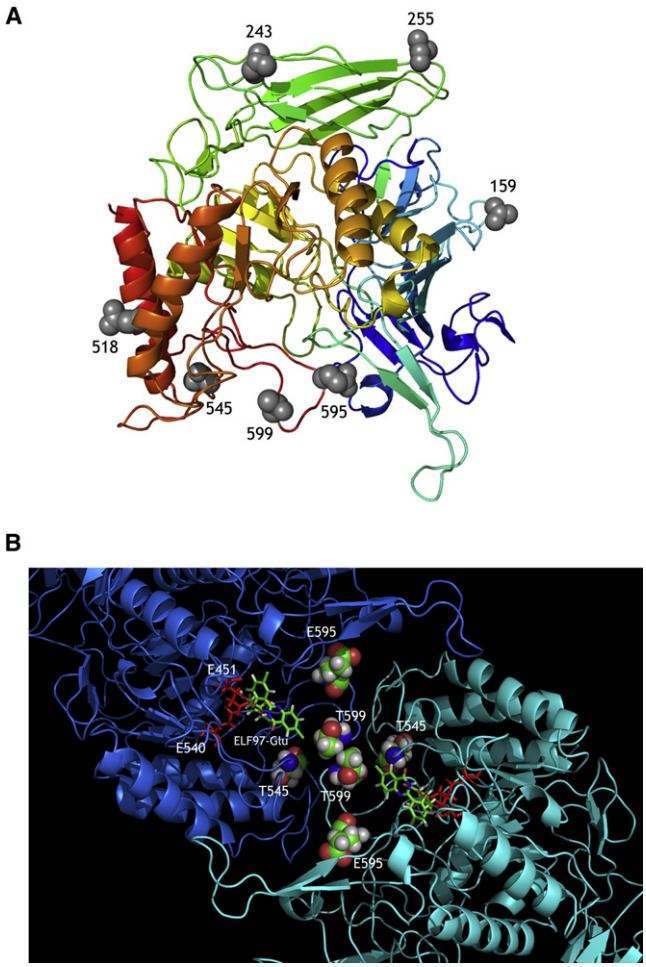


**Figure 5. Mutations Involved in Enhanced Enzyme Activity at Neutral pH Values**

(A) Amino acid changes in the hβG variants are represented as gray spheres. (B) Docking model of ELF-97 β-D-glucuronide present in two active sites formed by interactions of two hβG monomers, colored dark and light blue, respectively. The catalytic E451 and E540 residues are represented in red. ELF-97 β-D-glucuronide and T545, E595, and T599 residues are represented with green for carbon atoms, dark blue for nitrogen atoms, red for oxygen atoms, and gray for hydrogen atoms. The figure was prepared with PyMOL (http://pymol.sourceforge.net/).

The hβG variants identified in our study include amino acid substitutions at positions 159, 243, 255, and 518 on the surface of hβG and positions 545, 595, and 599 near the catalytic pocket (Figure 5A) (Islam et al., 1999). The catalytic site of hβG is formed by an α/β TIM barrel consisting of eight alternating parallel β strands and α helices. The top of the barrel is formed by loops connecting the C termini of the β strands to the N termini of the α helices. Two of the loops (β4α4 and β7α7) harbor the catalytic residues of hβG (E451 and E540, respectively), in common with many enzymes possessing TIM barrels (Altamirano et al., 2000). In addition, the β7α7 and β8α8 loops are involved in direct interactions with the corresponding loops in a neighboring monomer that form a large cavity harboring two active sites (Figure 5B) (Jain et al., 1996). T599 in the β8α8 loop interacts with the same amino acid in the neighboring subunit. Single amino acid

substitutions in E1-S (T545S) and E1-G (T545G) resulted in substantially enhanced substrate affinity. Interestingly, eβG variants that displayed enhanced catalytic activity against β-D-galactoside substrates also incorporated serine or alanine substitutions at the corresponding threonine (T509 in eβG) (Matsumura and Ellington, 2001), suggesting that amino acids with smaller side chains might increase the conformational flexibility of the β7α7 loop and alter substrate affinity. Replacement of T599 with hydrophobic amino acids was associated with increased βG activity at neutral pH, suggesting that alterations in monomer interactions may alter substrate binding. The negatively charged glutamic acid residue at position 595 was replaced by neutral or positively charged amino acids in many hβG variants, suggesting that removing a negative charge in the binding pocket might increase enzyme affinity for the negatively charged β-D-glucuronide ($pK_a$ ~2.8).

Clone E2-20, which includes two mutations on the enzyme surface (L243Q and L255Q), displayed about a 3-fold smaller $K_m$ as compared to E1-G, which shares a common mutation of T545G. S2 and S28 variants also have mutations in surface amino acids (V159M and Q518R, respectively). Q518 is present on the α6 helix of the TIM barrel. Substitution of glutamine with arginine is unlikely to disrupt the structure of the α helix, suggesting that the effect of this substitution was mediated by charge effects. Beneficial mutations present on the surface of hβG are difficult to explain, due to their distance from the active site. However, previous studies demonstrated that modifying surface amino acids can shift the pH profile of enzymes (Russell and Fersht, 1987). Our results confirm that amino acid substitutions which are distant from the catalytic cavity can contribute to enhanced βG activity.

We examined several approaches to increase the activity of hβG at neutral pH during the initial phase of this investigation. In contrast to hβG, eβG catalyzes the hydrolysis of glucuronides with high efficiency at pH 7.0. However, the structure and amino acids in the catalytic pocket of eβG and hβG are nearly identical, hindering rational design of hβG variants by comparison with eβG. On the other hand, molecular modeling indicated that the surface charge of eβG is more negative than hβG (Figure S2). Differences in the surface charge of enzymes may affect the $pK_a$ of catalytic amino acids, resulting in alterations in enzymatic pH optima (Russell and Fersht, 1987). Acid-base catalysis generally requires two carboxy side chains, one which is protonated and the other ionized. In hβG, E451 acts as an acid-base whereas E540 functions as a nucleophile (Islam et al., 1999). We hypothesized that reducing the surface charge of hβG might increase the $pK_a$ of E451, thereby maintaining the carboxy side chain in the acid form at elevated pH levels. Positive and neutral amino acids with side chains predicted to project away from the surface of hβG were therefore substituted with negatively charged amino acids. However, altering the surface charge had minimal effect on the pH optimum of hβG (Figure S3). In addition, all mutants displayed at least 85% reductions in catalytic activity (results not shown). In another approach, we attempted to identify amino acids important in controlling the pH profile of hβG by employing random chimeragenesis on transient templates (RACHITT) to shuffle eβG and hβG genes (Coco et al., 2002). We could not successfully shuffle these genes, however, likely due to their low overall homology of 50.7%.

In summary, we developed a mammalian cell-surface display approach to evolve hβG variants with enhanced capability to hydrolyze anticancer glucuronide prodrugs at the physiological conditions of the tumor. These hβG variants are anticipated to improve the efficacy of ADEPT and GDEPT and may allow multiple treatment rounds due to their low immunogenicity. Moreover, this technology may be applied on other enzymes and other selection conditions, as mammalian expression libraries of membrane-tethered enzymes allow straightforward in vitro evolution of enzyme properties.

## SIGNIFICANCE

**Many proteins require posttranslational modifications, such as glycosylation, to correctly fold and display biological activity. Development of high-throughput screening methods for identification of glycoproteins with altered properties will facilitate elucidation of protein structure-function relationships as well as allow isolation of improved proteins for novel biotechnological and medical applications. Toward this goal, we developed a high-throughput screening procedure for enzymatic activity based on tethered enzyme expression and stable retention of fluorescent reaction products in mammalian cells. A key advantage of this methodology is the ability to precisely control screening conditions such as reaction pH. Furthermore, fluorescence-activated cell sorting allows screening of $10^7$ enzyme variants per hour and reduces protein expression bias by simple quantitative measurement of surface expression levels. We demonstrated the utility of this screening procedure by isolating variants of human β-glucuronidase, a tetrameric glycoprotein normally present in lysosomes, that displayed significantly enhanced enzymatic activity at pH 7.0. The human β-glucuronidase variants isolated in our study are anticipated to extend the utility of selective cancer therapy by glucuronide prodrug treatment due to low immunogenicity in humans and enhanced catalytic activity at the physiological pH present in human tumors. The screening methodology described here should greatly facilitate investigation and modification of eukaryotic enzymes requiring posttranslational modifications for biological activity.**

## EXPERIMENTAL PROCEDURES

### Reagents and Antibodies

ELF-97 alcohol and ELF-97 β-D-glucuronide were from Molecular Probes (Eugene, OR, USA). 4-methylumbelliferyl β-D-glucuronide and CPT-11 were from Sigma-Aldrich (St. Louis, MO, USA). Biotinylated anti-HA antibody was from Vector Laboratories (Burlingame, CA, USA). Rhodamine-conjugated streptavidin and goat anti-rat FITC conjugate were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Mouse anti-hβG mAb 7G8 and rat anti-mouse βG (mβG) mAb 7G7 have been described (Chen et al., 2007). 7G8 was directly labeled with FITC as described (Goding, 1976). HAMG was synthesized as described (Roffler et al., 1991). SN-38G was purified by HPLC from the urine of mice treated with CPT-11.

### Cells

BALB/3T3 fibroblasts (CCL-163; ATCC, Manassas, VA, USA) and CT26 murine colon carcinoma cells (ATCC CRL-2638) were cultured in DMEM (4.5 g/l glucose) supplemented with 10% bovine serum, 2.98 g/l HEPES, 2 g/l $NaHCO_3$, 100 U/ml penicillin, and 100 μg/ml streptomycin. EJ human bladder carcinoma

cells (Marshall et al., 1977) were cultured in RPMI containing the same supplements. CT26 and EJ cell lines expressing membrane-tethered mβG on their surface have been described (Chen et al., 2007). All cells were free of mycoplasma as determined by PCR.

### Error-Prone PCR

A silent mutation was made to change cytosine to guanidine at position 411 of the hβG gene in pLNCX-hβG-eB7 (Chen et al., 2007) to remove an internal SalI site. The resulting vector, pLNCX-hβGs-eB7, was employed as a template for error-prone PCR (Cadwell and Joyce, 1992) using primer P1 (5′-TAT GCT GGG GCC CAG CCG GCC-3′), which contains part of the HA epitope at the 5′ end and an SfiI restriction site at the 3′ end (underlined), and primer P2 (5′-CTG AGA TGA GTT TTT GTT CGT CGA C-3′), which contains part of the myc epitope at the 5′ end and a SalI restriction site at the 3′ end (underlined). Mutagenic buffer (8 mM dCTP, 8 mM dTTP, 48 mM $MgCl_2$, 5 mM $MnCl_2$) (Matsumura and Ellington, 2001) was added (1.25 or 2.5 μl) to each 50 μl PCR reaction using 5 units *Taq* polymerase (Takara, Shiga, Japan) for amplification. The PCR product was digested with SfiI and SalI enzymes, ligated into the same sites in pLNCX-hβGs-eB7, and transformed into DH5α competent cells by electroporation. Transformed bacteria were selected on 15 cm carbenicillin-containing LB agar plates for 16 hr at 37°C. Colonies from multiple plates were collected and expanded in carbenicillin-containing LB medium and then amplified by addition of 170 μg/ml chloramphenicol. Plasmid DNA from each library was purified by centrifugation in a CsCl-ethidium bromide density gradient at 60,000 rpm in a Ti 70.1 rotor at 4°C for 24 hr using a Beckman Optima L-90K ultracentrifuge (Beckman Coulter, Fullerton, CA, USA).

### Retroviral Transduction

To generate stable cell libraries or His-tagged soluble hβG producer cells, plasmid DNA was cotransfected with pVSV-G (Clontech, Mountain View, CA, USA) into GP293 cells (Clontech) to produce recombinant retroviral particles. Two days after transfection, the culture medium was filtered, mixed with 8 mg/ml polybrene, and added to 3T3 fibroblasts. Stable cell lines were selected in medium containing 0.5 mg/ml G418 (Calbiochem, San Diego, CA, USA).

### Selection of High-Activity hβG Cells

3T3 cell libraries were screened by fluorescence-activated cell sorting (FACS). Typically, $10^7$ 3T3 cells expressing membrane-tethered hβG variants were washed and suspended in 0.4 ml BSA/HBSS (5.4 mM KCl, 0.3 mM $Na_2HPO_4$, 0.4 mM $KH_2PO_4$, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM $MgSO_4$, 137 mM NaCl, 5.6 mM D-glucose [pH 7.4]) containing 0.5% BSA and 20 μg/ml 7G8-FITC for 30 min at 4°C. Cells were then washed and incubated with 25, 50, or 100 μM ELF-97 β-D-glucuronide at defined pH values in 50 mM bis-Tris, 25 mM glucose, 85.6 mM NaCl, 5.4 mM KCl, 0.6 mM $MgSO_4$, 1.3 mM $CaCl_2$ at 37°C for 10 min. The cells were washed with ice-cold 0.5% BSA/HBSS and suspended in 0.5% BSA/HBSS containing 5 μg/ml propidium iodide (Sigma). Cells were sorted on a FACSVantage DiVa (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with an Enterprise IIC argon laser for dual excitation at 488 and 351–364 nm. Dead cells (propidium iodide positive, high FL3 fluorescence) were gated out before 7G8-FITC immunofluorescence was detected at excitation/emission wavelengths of 488/515 nm (FL1) and ELF-97 alcohol was detected at excitation/emission wavelengths of 351/530 nm (FL4). In later experiments, cells were sorted twice each round; cells exhibiting the highest 10% activity were collected and then immediately sorted again to collect the cells displaying the highest 5% activity (representing 0.5% of the total starting population). Double sorting greatly decreased contamination with low-activity cells. The sorted cells were cultured for 4–10 days for sequential rounds of cell sorting or RNA extraction. Flow cytometer data were analyzed using Flowjo (Tree Star, Ashland, OR, USA).

### DNA Shuffling

Variant hβG genes were recovered by RT-PCR from the cells collected after three rounds of FACS. hβG DNA (1 μg) was digested with 0.025 U of DNase I (Takara) in 25 μl of 50 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$ for 11 min at 25°C. The reaction was quenched by adding 5 μl of 0.5 M EDTA. DNA fragments ranging from 100 to 300 bases on a 1% agarose gel were reassembled in 35 cycles of primerless PCR (Stemmer, 1994). The full-length recombinant products, amplified in a standard PCR reaction with P1 and P2 primers, were cloned in pLNCX-hβGs-eB7 to create a new 3T3 cell library.

### Saturation Mutagenesis

To generate randomized mutations at amino acid positions 545, 595, and 599 in hβG, two rounds of site-directed mutagenesis were performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). All possible amino acids were introduced at amino acid position 545 with primers P3 (5′-CGA GTA TGG AGC AGA ANN SAT TGC AGG GTT TCA CCA GGA TCC-3′) and P4 (5′-GGA TCC TGG TGA AAC CCT GCA ATS NNT TCT GCT CCA TAC TCG-3′), where N represents G, A, T, or C, and S represents G or C. A second round of site-directed mutagenesis was performed with primers P5 (5′-GGA ATT TTG CCG ATT TCA TGA CTN NSC AGT CAC CGN NSA GAG TGC TGG GGA ATA AAA AGG GG-3′) and P6 (5′-CCC CTT TTT ATT CC CAG CAC TCT SNN CGG TGA CTG SNN AGT CAT GAA ATC GGC AAA ATT CC-3′) to further introduce all possible amino acids at positions 595 and 599 of hβG. The resulting saturation mutagenesis DNA library was ligated into the SfiI and SalI sites present in pLNCX-hβGs-eB7 and employed to generate a 3T3 cell library.

### Purification of Recombinant hβG Proteins

Recombinant His-tagged hβG was purified from stable 3T3 fibroblast lines as described (Wu et al., 2004).

### Enzyme Assays

The pH-dependent enzyme activities of recombinant hβG variants were measured in triplicate with 0.1 mM ELF-97 β-D-glucuronide at 37°C for 30 min at defined pH ranging from 3 to 10 in βG reaction buffer (50 mM bis-Tris, 50 mM triethanolamine, 100 mM acetic acid, 0.1% BSA). The reaction was quenched by adding an equal volume of stop buffer (2 M Tris-HCl, 0.8 M sodium bicarbonate [pH 8]). The fluorescence of ELF-97 alcohol and 4-methylumbelliferone was measured in a Gemini EM microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA) at excitation/emission wavelengths of 355/555 nm. Kinetic values for hβG substrate hydrolysis were determined by diluting ELF-97 β-D-glucuronide (2 mM) in pH 4.5 or pH 7.0 βG reaction buffer 1:1 with defined concentrations of hβG in 200 μl. Fluorescence was immediately measured under thermal control at 37°C for 8–10 min. The measurement was repeated using the same amount of enzyme for different concentrations of substrate in an optimal range. The acquired readings were converted to product concentration by preestablished standard curves. Double reciprocal plots were used to determine $K_m$ and $k_{cat}$. Kinetic assays were performed at least three times and mean values were calculated. The stability of recombinant hβG variants was assayed by measuring their enzymatic activity at time 0 and again after incubating 5 μg of purified enzyme in PBS (containing 0.5 mg/ml BSA) at 37°C for 14 days. The enzymatic activities of the hβG variants were measured in triplicate with 0.5 mM 4-methylumbelliferyl β-D-glucuronide at 37°C for 15 min at pH 7.0 in βG reaction buffer. The reaction was terminated by adding an equal volume of stop buffer (1 M glycine, 0.5 M sodium bicarbonate [pH 11]) and the fluorescence was measured at excitation/emission wavelengths of 355/460 nm in a microplate spectrofluorometer.

### [$^3$H]Thymidine Incorporation Assay

Defined concentrations of purified recombinant hβG were added with 10 μM *p*-hydroxyaniline mustard glucuronide (HAMG) or 100 nM SN-38 glucuronide (SN-38G) to 5000 EJ human bladder cancer cells in 200 μl of complete medium at pH 6.8 for 24 hr. The cells were washed and incubated in fresh medium for 24 hr and then pulsed for 16 hr with [$^3$H]thymidine (1 μCi/well). The cells were harvested and the radioactivity was measured in a TopCount microplate scintillation counter (Packard, Meriden, CT, USA). Results are expressed as percent inhibition of [$^3$H]thymidine incorporation into cellular DNA in comparison to untreated cells.

### Molecular Modeling

To examine potential interactions of ELF-97 β-D-glucuronide with mutated amino acids in hβG variants, the X-ray crystal structure of eβG with glucaro-1,5-lactone in the active site (unpublished data) was employed as a template to position the glucuronide group of ELF-97 β-D-glucuronide. ELF-97

β-D-glucuronide was then docked into the active sites formed by the interaction of two hβG monomers using Molegro 2.2.0 (Aarhus, Denmark).

### Statistical Analysis

Statistical significance of differences between mean values was estimated by Excel (Microsoft, Redmond, WA, USA) using the independent t test for unequal variances. P values of less than 0.05 were considered to be statistically significant.

## SUPPLEMENTAL DATA

Supplemental Data include three figures and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(08)00411-0/.

## ACKNOWLEDGMENTS

This work was supported by a grant from the National Science Council, Taiwan (NSC-95-2311-B001-068-MY3). We thank Wen-Wen Chen for assistance with fluorescence cell sorting.

Received: July 1, 2008
Revised: September 8, 2008
Accepted: October 20, 2008
Published: December 19, 2008

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